IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| DECLARATION UNDER 37 C.F.R. §1.132 | | ATTORNEY DOCKET NO. 09680.0352US01 | |
|---|---|---|---|
| | | U.S. APPLICATION SERIAL NO. 10/019,931 | CONFIRMATION NO 8743 |
| | | FILING DATE May 10, 2002 | |
| INVENTOR(S) Luc VARIN et al. | EXAMINER Stuart F. Baum | | GROUP ART UNIT 1638 |
| TITLE OF APPLICATION METHOD, COMPOSITIONS, AND GENETIC SEQUENCES FOR MODULATING FLOWERING PLANTS, AND PLANTS GENETICALLY MODIFIED TO FLOWER EARLY AND TARDILY | | | |

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Luc Varin, hereby declare as follows:

1. I received a Ph.D. in Biochemistry from Concordia University in 1990;

2. I am currently an Associate Professor of the Biology Department at Concordia University, 1455 West De Maisonneuve Blvd., Montreal Qc H3G 1M8;

3. I have been teaching Molecular Biology and Agricultural Biotechnology courses in the Biology Department at Concordia University since 1996;

4. I am familiar with the Official Action of May 17, 2006, on this application.

5. A person skilled in the art would use the term "sequence similarity" to refer to the presence of an identical or conserved amino acid at the same position when two sequences are compared. A person skilled in the art would use the term "conserved" to refer to amino acid having similar chemical properties (e.g lysine and arginine for positively charged amino acids; aspartate and glutamate for negatively charged amino acids; serine and cysteine because they have more or less the same size and that they can form hydrogen bonding; etc.). A person skilled in the art would use the term "sequence identity" to refer to the presence of the same nucleotide or amino acid at the same position when two sequences are compared. The terms “sequence similarity,” “conserved” and “sequence identity” were all well-known by those skilled in the art at the time the present application was filed.

However, it is common to see in the literature the term "similarity" in replacement for "identity" when comparing nucleic acids. In the context of the present application, the term "sequence similarity is used within the definition of the term "functional homologue" and would be understood by a person skilled in the art as referring to conserved amino acids.

6. As used within the present invention, a person skilled in the art would understand the "functional homologue" to two or more sequences having the same biochemical or biological function even though they do not have identical sequences. In the context of the present invention, such function is a catalytic function i.e. the sequences catalyze the same reaction (sulfonation) on the same substrate (12-hydroxyjasmonate). Hence, a person skilled in the art would easily understand that the functional homologues according to the present inventions are sequences having the function of an 11- or 12- hydroxyjasmonate sulfotransferase.

7. "High stringency conditions" are easily determined by a person skilled in the art. Such a person would refer, for instance, to a manual of molecular biology such as chapter 9 of Molecular Cloning: A Laboratory Manual from Sambrook, Fritsch and Maniatis (ISBN: 087969-309-6, Cold Spring Harbor Laboratory publisher) to determine stringency conditions of hybridization and how to modify them.

8. *Arabidopsis* has been widely recognized as a model plant as shown for instance in the following United States Department of Agricultural Research Service websites:

http://www.ars.usda.gov/is/AR/archive/jan99/dopsis0199.htm and

http://www.nsf.gov/pubs/2002/bio0202/2010report_8.pdf

9. In order to address the Examiner's rejection of claims 59-73 under 35 USC 112 1st and 2nd paragraph, the following is provided below:

## EXAMPLE 1: MOLECULAR CLONING OF *BNST2A* AND PRODUCTION OF *BRASSICA NAPUS* PLANTS THAT FLOWER EARLY OR TARDILY.

In the first example, the cDNA encoding *AtST2a* (SEQ ID NO.1) was used to screen a genomic library from *Brassica napus*. This work was done according to well-known procedures described in chapter 9 of Molecular Cloning: A Laboratory Manual from Sambrook, Fritsch and Maniatis (ISBN: 087969-309-6, Cold Spring Harbor Laboratory publisher). The hybridization was done at high stringency and allowed to select one genomic clone containing the full-length sequence of *BnST2a*. A researcher facing a negative result at high stringency would modify the experimental conditions until a positive hybridization signal is obtained. The modifications are described in the book cited previously and are common procedures for this type of work and are considered routine.

The genomic clone was sequenced and the open reading frame encoding *BnST2a* could be retrieved easily by sequence comparison with *AtST2a*. The coding sequence was subcloned in the bacterial expression vector pQE30 (Qiagen) and the protein expressed in *E. coli*. The recombinant protein was assayed with 12-hydroxyjasmonate as substrate using a radioactivity-based assay with [$^{35}$S]-3'-phosphoadenosine 5'-phosphosulfate as co-substrate. The protein was found to be specific for hydroxyjasmonates.

The coding sequence of *BnST2a* was introduced in the plant transformation vector pRD400 in the sense and antisense orientation. Transgenic *Brassica napus* were produced according to the procedure described by (MM Moloney, JM Walker, KK Sharma, (1989) High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Reports 8:238-242). Transgenic lines harboring a single insertion of the T-DNA were selected and analyzed for flowering time. The experiment was conducted under two different photoperiods. In the first one, plants were initially grown in growth chambers under long photoperiods (16 hours of light). After flowering, they were transferred to the greenhouse. In the second one, plants were grown in the greenhouse for the entire length of the experiment (8-11 hours of light).

For each experiment, two sets of plants were used. The first set consisted of 6 different lines of transgenic plants expressing *BnST2a* in the sense orientation. The second set consisted of 5 different lines expressing the *BnST2* cDNA in the antisense orientation.

### a) <u>First set of plants expressing *BnST2a* in the sense orientation</u>

Six lines were selected and 20 plants were grown per line. 10 plants were grown under short photoperiod while 10 plants were grown under long photoperiod. An additional 20 wild type plants were grown under the same conditions as described above. For each plant, a Western blot was conducted to confirm the presence or the absence of the *AtST2a* protein.

**Long photoperiod (16 hours)**

Plants were grown in growth chambers at Concordia University. This experiment was conducted at a temperature of 25°C and a relative humidity of 40%.

Table 1 shows the results of all the plants of the six lines as compared to the wild type plants. A difference of 10 days is observed between the flowering time transgenic plants as compared with wild type plants. Flowering time was measured as the first appearance of the flower bud. However, no significant difference can be observed between the transgenic and wild type plants for the number of flowers, siliques and wet weight of the plants.

**Table 1**

| Plants | Flowering time (days) | Number of flowers | Number of fruits (siliques) | Wet weight (grams) |
|---|---|---|---|---|
| Transgenics* | 47 (± 6.7) | 401 (± 138) | 182 (± 66) | 149 (± 34) |
| Control** | 37 (± 7.8) | 365 (± 82) | 193 (± 70) | 138 (± 14) |

* N=59 The 59 plants tested positive in Western blot
** N=10 The 10 plants tested negative in Western blot

**Table 2 shows the average flowering time per line.**

| Transgenic line | Flowering time in days* |
|---|---|
| 3.4 | 44.5 |
| 3.7 | 44 |
| 3.11 | 48.3 |
| 2.4 | 51.3 |
| 2.6 | 48.5 |
| 2.7 | 47.5 |
| Control | 36.6 |

* Average flowering time per line N = 10

Line 2.4 is the most affected with a mean flowering time of 51 days as compared with 37 days for the control plants.

**Short photoperiod (8-11 hours)**

Plants were grown in the greenhouse at Concordia University. The photoperiod varied between 8 and 11 hours. The temperature varied from 20 to 30°C. No additional growth lamps were used during this experiment to mimic field conditions. The relative humidity varied from 35 to 55%.

Table 3 shows the results of all the transgenic plants of the five lines as compared with the wild type plants. No significant difference is observed for all the parameters that were studied.

**Table 3**

| Plants | Flowering time (days) | Number of flowers | Number of siliques | Wet weight (grams) |
|---|---|---|---|---|
| Transgenics* | 77 (± 10.9) | 1588.4 (± 401) | 584 (± 156) | 329 (± 60) |
| Control** | 73 (± 7.3) | 1606 (± 481) | 573 (± 150) | 324 (± 53.5) |

* N=57 The 57 plants tested positive in Western blot
** N=10 The 10 plants tested negative in Western blot.

b) **<u>Second set of plants expressing *BnST2a* in the antisense orientation</u>**

These lines express the *BnST2* gene from *Brassica napus* in the antisense orientation. Five lines were selected and 20 plants were grown per line. 10 plants were grown under short photoperiod while 10 plants were grown under long photoperiod. An additional 20 wild type plants were grown under the same conditions as described above. A PCR reaction was conducted on the genomic DNA extracted from each plant to confirm for the presence or absence of the transgene.

**Long photoperiod (16 hours)**

This experiment was conducted at a temperature of 25°C and a relative humidity of 40%

Table 4 shows the results of all the plants of the five lines as compared to the wild type plants. No significant difference can be observed between the transgenic and wild type plants for all the parameters that were measured.

**Table 4**

| Plants | Flowering time (days) | Number of flowers | Number of siliques | Wet weight (grams) |
|---|---|---|---|---|
| Transgenics* | 39 (± 5.9) | 505 (± 229) | 272 (± 96) | 146 (± 39) |
| Control** | 37 (± 7.8) | 365 (± 82) | 193 (± 70) | 138 (± 14) |

* N=49 Positive PCR results were obtained for all the plants
** N=10 Negative PCR results were obtained for al the plants. Same plants as the control in table 1

**Short photoperiod (8-11 hrs)**

Plants were grown in a greenhouse and the photoperiod varied between 8 and 11 hours. The temperature varied from 20 to 30°C. The relative humidity varied from 35 to 55%.

Table 5 shows the results from all the plants of the five lines as compared to the wild type plants. A significant difference is observed in the flowering time of the transgenic plants as compared with the control. Although not significant, there is a trend for a reduced

number of flowers in the transgenic plants. However, the total number of siliques (containing the seeds) seems not affected.

**Table 5**

| Plants | Flowering time (days) | Number of flowers | Number of siliques | Wet weight (grams) |
|---|---|---|---|---|
| Transgenic* | 80 (± 10.6) | 1045 (± 443) | 681 (± 329) | 302 (± 118) |
| Control** | 91 (± 12.7) | 1324 (± 465) | 550 (± 195) | 301 (± 82) |

* N=49 Positive PCR results were obtained for all the plants
** N=10 Negative PCR results were obtained for al the plants

**Table 6 shows the average flowering time per line**

| Line | Flowering time in days* |
|---|---|
| 1.2 | 76.4 |
| 5 | 92.9 |
| 10 | 73 |
| 20 | 84.7 |
| 23 | 74.6 |
| Control | 91 |

* Average flowering time per line N = 10

Line 10 is the most affected with an average flowering time of 18 days earlier than wild type plants.

**Summary**

The results are similar to the ones obtained with *Arabidpsis thaliana* and described in the patent application 10/019.931.

- A delayed flowering phenotype is observed in plants overexpressing *BnST2a* when grown under inductive photoperiod (long day),
- An early flowering phenotype is observed in plants overexpressing *BnST2a* in the antisense orientation when growing under non-inductive photoperiod (short day).

Jasmonate levels were not quantified in the transgenic *Brassica* lines. However, the present patent application no. 10/019,931 demonstrates that as expected, the levels of 12-

hydroxyjasmonate and its sulphated derivatives are modified in transgenic *Arabidopsis thaliana* expressing *AtST2a* in sense and antisense orientation.

This example illustrates how a researcher skilled in the art of plant molecular biology can easily use the information present in the present application, U.S. Serial No. 10/019,931, to isolate a homologue of *AtST2a* and use it to generate transgenic lines having altered flowering time in another plant species.

## EXAMPLE 2: SEARCH FOR GENES ENCODING 12-HYDROXYJASMONATE SULFOTRANSFERASES FROM OTHER PLANT SPECIES USING EST AND GENOMIC DATABASES

The amino acid sequence described in the present application, U.S. Serial No. 10/019,931, contains the motif that is well-known to be present in all soluble sulfotransferases that have been characterized so far. It is possible to easily find this information by doing a protein blast search at NCBI. More specifically, two domains are highly conserved.

- The first one comprises the sequence YPKSGTTW and is localized at the N-terminal of all sulfotransferases. The lysine residue of this domain has been shown to bind the sulfate donor 3'-phospoadenosine 5'-phosphosulfate (see Marsolais, F and Varin, L. (1995) Identification of amino acid residues critical for catalysis and substrate binding in the flavonol 3-sulfotransferase. *J. Biol. Chem.*, 270, 30458-30463.)
- The second domain, RKXXGDWKNXFT, is localized closer to the C-terminal extremity of all sulfotransferases. The arginine residue of this motif is critical for the binding of the sulfate donor.
- There are also other amino acid residues that have been shown to be absolutely required for activity such as histidine 118 (numbering of the flavonol 3-sulfotransferase), which acts as base catalyst during catalysis.

It is very easy for one skilled in the art, such as a researcher with expertise in protein chemistry, to find these conserved domains and to assess the sulfotransferase function in an unknown protein having these motifs. Another reference that is known to such a person in the art and that can be used to find the structural characteristics of sulfotransferases is: Marsolais, F. and Varin, L. (1998) Recent developments in the study of the structure-function relationship of flavonol sulfotransferases. *Chem. Biol. Interact.*, 109, 117-122.

In the second example, it is demonstrated how one skilled in the art can easily retrieve 12-hydroxyjasmonate sulfotransferase-coding sequences by applying simple procedures that are well known to such a person:

- First, sulfotransferase-coding genes are easily retrieved by doing a blast search of the GeneBank database at NCBI (http://www.ncbi.nlm.nih.gov/). The query sequence used for this search can be *AtST2a* or any other sulfotransferase characterized previously. The search can be restricted to plant sequences. The search can also be done in the TIGR databases for plant sequences (http://www.tigr.org/plantProjects.shtml). Using this approach, I could retrieve more than 200 sequences. At this stage all sulfotransferase-coding genes are retrieved. The search as to be refined to focus on sequences that can code for hydroxyjasmonate sulfotransferases.
- To find sequences closely related to *AtST2a*, a phylogenetic tree is generated. The tree will allow identifying proteins belonging to the same clade. The figure bellow illustrates the result of this approach.

This example demonstrates that a person skilled in the art will be lead without difficulty to predict the function of a sulfotransferase by comparing its sequence to *AtST2a*. Furthermore the example defines the metes and bounds of proteic sequences having the same function as the protein *AtST2a*.

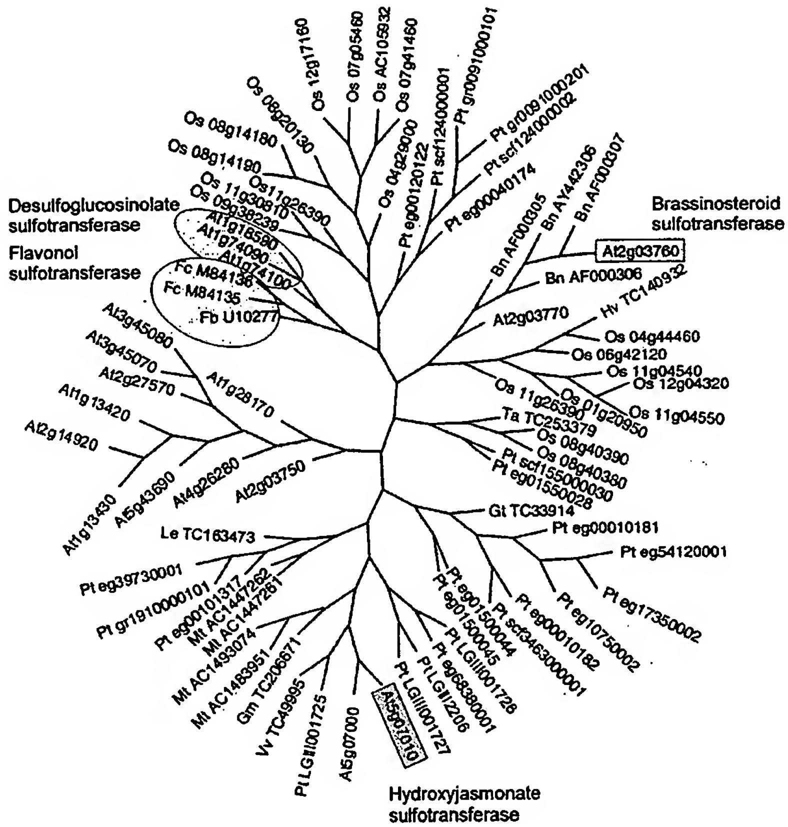


Figure legend. Cladogram showing the relationship between 78 plant sulfotransferases sequences. The accession number is preceded by the initial of the plant species in which they were identified. Abbreviations for species: At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Fb, *Flaveria bidentis*; Fc, *Flaveria chloraefolia*; Gm, *Glycine max*; Gt, *Gentiana triflora*; Hv, *Hordeum vulgare*; Le, *Lycopersicon esculentum*; Mt, *Medicago truncatulata*; Os, *Oryza sativa*; Pt, *Populus trichocarpa*; Ta, *Triticum aestivum*; Vv *Vitis vinifera*. The *Arabidopsis* and *Flaveria* genes which were characterized at the biochemical level are highlighted. The sequences were aligned with ClustalW and the cladogram was generated using PAUP 4.0b10 (Ativec) by a neighbor joining method.

The sequences used to generate the cladogram presented above were selected according to the following criteria. First, the regions I to IV conserved in all cytosolic sulfotransferases had to be present in the sequence (Varin et al., 1992, PNAS USA 89:1268-90). Second, only sulfotransferases that could be assembled into full-length sequences were included in the alignment and in the phylogenetic tree. Cladograms from the alignment of the conserved region I or conserved region IV were also generated. In all cases, the trees looked similar to the one presented above.

To date, all monocotyledonous and dicotyledonous plants that were tested were found to accumulate sulfated hydroxyjasmonates indicating that the sulfotransferase enzyme involved in their synthesis is conserved among species. Recently, the *A. thaliana* hydroxyjasmonate sulfotransferase (*AtST2a*) was shown to be encoded by the locus *At5g07010* (Gidda et al. 2003, The Journal of Biological Chemistry, 278:17895-17900 ). This enzyme is located in a clade comprising members from poplar, grape, alfalfa and soybean (Figure above). The first and second closest relatives of *A. thaliana* hydroxyjasmonate sulfotransferase are from poplar (pt_LGIII0001725) and grape (VvTC49995) with 60% and 58% amino acid identity, respectively. Annex 1 presents the results of the amino acid sequence comparison of *AtST2a* with the *Brassica*, poplar and grape vine enzymes.

The coding sequence of the poplar cDNA was introduced in the bacterial expression vector pQE30 and assayed for 12-hydroxyjasmonate sulfotransferase activity. The results demonstrated that the poplar enzyme exhibits the same specificity as *AtST2a*. The grape vine enzyme was not tested but one can easily predict that similar results would be obtained. In front of a new sequence, a person skilled in the art could easily apply the same approach to determine whether if the new sequence falls in the same clade as *AtST2a*. Alternatively, one skilled in the art could subclone without difficulty the sequence in a vector and assay its activity.

**Summary**

In conclusion, the second example demonstrates that one skilled in the art of molecular biology could easily apply available tools to screen for enzymes having the same function as *AtST2a* from a large collection of sequences. The results also demonstrates that the poplar enzyme with 60% amino acid sequence identity has the same function as *AtST2a* (see Annex 1).

The two examples above describe how one skilled in the art of molecular biology could easily use the information described in the present application U.S. Serial No. 10/019,931, to retrieve other sulfotransferase-coding genes having the same biochemical function as *AtST2a* (12-hydroxyjasmonate sulfotransferase). The two examples describe methodologies that are available to the entire scientific community and clearly to those skilled in the art.

Finally, Annex A (attached to the Amendment filed herewith) and Annexes 1 and 2 hereinbelow are all incorporated in the entirety in this Declaration.

**Annex 1**
**Sequences described in the text**

**AtST2a**
MATSSMKSIP MAIPSFSMCH KLELLKEGKT RDVPKAEEDE GLSCEFQEML DSLPKERGWR
TRYLYLFQGF WCQAKEIQAI MSFQKHFQSL ENDVVLATIP KSGTTWLKAL TFTILNRHRF
DPVASSTNHP LFTSNPHDLV PFFEYKLYAN GDVPDLSGLA SPRTFATHLP FGSLKETIEK
PGVKVVYLCR NPFDTFISSW HYTNNIKSES VSPVLLDQAF DLYCRGVIGF GPFWEHMLGY
WRESLKRPEK VFFLRYEDLK DDIETNLKRL ATFLELPFTE EEERKGVVKA IAELCSFENL
KKLEVNKSNK SIKNFENRFL FRKGEVSDWV NYLSPSQVER LSALVDDKLG GSGLTFRLS

**BnST2a**
MATSSIKSVP IMAIPSFSIC HKHELLKEEG KSRDPKRQEE EEEGLSYEFL EMLDSLPKER
GWRTRHLYLF QGFWCQAKEI HAIMSFQKHF KTLPKDVILA TIPKSGTTWL KALTFTLLNR
HRFDPVSDHP LLTSNPHDLV PFLEYKLYAN GEVPDLSGLA SPRTFATHVP FGSLKGSIEE
PGAKVVYLCR NPFDTFISSW HFSNSIKSES VSPVSLEEGF DLYCRGVIGF GPFWEHMLGY
WRESLERPEK VLFLKYEDLK EDIETNLKKL ASFLGVSFTE EEEVKGVVKA VADLCSFESL
KKLEVNKSNK SIKNFENRYL FRKGEVSDWR NYLSPVQVER LSALVDDKLG GSGLTFRYC

**pt_LGIII0001725**
MVLNHFTKNQANDNGEDLERLTNECKELLLSLPREKGWRTACLYKYKGFWCQPKEIQAIISFQKH
FEPRDTDVILASIPKSGTTWLKALSFAILNRKKFAISSNDHPLLVSNPHDLAPFFEYKLYADKQV
PDLSKLPDPRLFATHIPFASLQDSIKKSNCRIIYICRNPFDTFISSWTFSNKLRSETVPPLLLEE
TFKMYCEGVVGFGPFWDHMLGYWKESLERQDKVLFLKYEDMKADVTFYLKKIAKFLGCPFSMEEE
KEGVVEKIASLCSFEKMKNLEVNKSGRSITNFENKHLFRKAEVGDWVNYLSPSMVKQLSQLIEEK
LGGSGIEFKVFP

**VvTC49995**
MGRTQFPKSQSAAEDAEELSHECKELLLSLPKERGWRTPHIYLYQGFWCQPKEIQAITNFQHHFQ
ARDTDLILATMPKAGTTWLKAMAFAILNRKPIPVSTTHPLHTSNPHDLVPFLEYKLYANNNFPDL
SKLPQPRLFATHVPFASLPESITKSNSRVVYMCRNPLDAFASSWHFLMKARPESLGPISIEEGFQ
MYCKGSMAFGPFWKHMLGYWKESIERPHKVLFMKYEDTKEDIIFQLKRLAKFLGVPFSLEEERKG
VIEEIARLCSFENLKNLEVNQSGKSIGYFENKDLFRKGVVGDWVNLLTPLMAKQLVQVMEEKLEG
SDLSFKVF

**Annex 2 Amino acid sequence comparison between *AtST2a* and proteins belonging to the same clade.**

**% aa identity**

| | *AtST2a* | *BnST2* |
|---|---|---|
| AtST2a | 100 | 83 |
| BnST2 | 83 | 100 |
| Pt_LGII001725 | 60 | 60 |
| Vvtc49995 | 58 | 59 |

**A) Amino acid sequence alignement of AtST2a with the 12-hydroxyjasmonate sulfotransferase from *Brassica napus* (BnST2a)**

```
AtST2a        1 MATSSMKSIP-MAIPSFSMCHKLELLKE-GKTRDVPKAEEDE-GLSCEFQEMLDSLPKER
BnST2a        1 MATSSIKSVPIMAIPSFSICHKHELLKEEGKSRDPKRQEEEEEGLSYEFLEMLDSLPKER

AtST2a       58 GWRTRYLYLFQGFWCQAKEIQAIMSFQKHFQSLENDVVLATIPKSGTTWLKALTFTILNR
BnST2a       61 GWRTRHLYLFQGFWCQAKEIHAIMSFQKHFKTLPKDVILATIPKSGTTWLKALTFTLLNR

AtST2a      118 HRFDPVASSTNHPLFTSNPHDLVPFFEYKLYANGDVPDLSGLASPRTFATHLPFGSLKET
BnST2a      121 HRFDPVS---DHPLLTSNPHDLVPFLEYKLYANGEVPDLSGLASPRTFATHVPFGSLKGS

AtST2a      178 IEKPGVKVVYLCRNPFDTFISSWHYTNNIKSESVSPVLLDQAFDLYCRGVIGFGPFWEHM
BnST2a      178 IEEPGAKVVYLCRNPFDTFISSWHFSNSIKSESVSPVSLEEGFDLYCRGVIGFGPFWEHM

AtST2a      238 LGYWRESLKRPEKVFFLRYEDLKDDIETNLKRLATFLELPFTEEEERKGVVKAIAELCSF
BnST2a      238 LGYWRESLERPEKVLFLKYEDLKEDIETNLKKLASFLGVSFTEEEEVKGVVKAVADLCSF

AtST2a      298 ENLKKLEVNKSNKSIKNFENRFLFRKGEVSDWVNYLSPSQVERLSALVDDKLGGSGLTFR
BnST2a      298 ESLKKLEVNKSNKSIKNFENRYLFRKGEVSDWRNYLSPVQVERLSALVDDKLGGSGLTFR

AtST2a      358 LS
BnST2a      358 YC
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**B) Amino acid sequence alignement of AtST2a with the 12-hydroxyjasmonate sulfotransferase from Poplar**

```
AtST2a              1 MATSSMKSIPMAIPSFSMCHKLELLKEGKTRDVPKAEEDEGLSCEFQEMLDSLPKERGWR
pt_LGIII0001725     1 ----------MVLNHFT-----------KNQANDNGEDLERLTNECKELLLSLPREKGWR

AtST2a             61 TRYLYLFQGFWCQAKEIQAIMSFQKHFQSLENDVVLATIPKSGTTWLKALTFTILNR
pt_LGIII0001725    40 TACLYKYKGFWCQPKEIQAIISFQKHFEPRDTDVILASIPKSGTTWLKALSFAILNR

AtST2a            118 HRFDPVASSTNHPLFTSNPHDLVPFFEYKLYANGDVPDLSGLASPRTFATHLPFGS
pt_LGIII0001725    97 KKFA--ISSNDHPLLVSNPHDLAPFFEYKLYADKQVPDLSKLPDPRLFATHIPFASL

AtST2a            174 LKETIEKPGVKVVYLCRNPFDTFISSWHYTNNIKSESVSPVLLDQAFDLYCRGVIG
pt_LGIII0001725   152 QDSIKKSNCRIIYICRNPFDTFISSWTFSNKLRSETVPPLLLEETFKMYCEGVVGF

AtST2a            230 FGPFWEHMLGYWRESLKRPEKVFFLRYEDLKDDIETNLKRLATFLELPFTEEEERK
pt_LGIII0001725   208 GPFWDHMLGYWKESLERQDKVLFLKYEDMKADVTFYLKKIAKFLGCPFSMEEEKEG

AtST2a            286 GVVKAIAELCSFENLKKLEVNKSNKSIKNFENRFLFRKGEVSDWVNYLSPSQVERL
pt_LGIII0001725   264 VVEKIASLCSFEKMKNLEVNKSGRSITNFENKHLFRKAEVGDWVNYLSPSMVKQLS

AtST2a.           342 SALVDDKLGGSGLTFRLS- 359
pt_LGIII0001725   320 QLIEEKLGGSGIEFKVFP 337
```

**C) Amino acid sequence alignement of AtST2a with the sulfotransferase from grape vine belonging to the same clade**

```
AtST2a          MATSSMKSIPMAIPSFSMCHKLELLKEGKTRDVPKAEEDEGLSCEFQEMLDSLPKERGWR 60
VVTC49995       ----------MGRTQFPKSQ-------------SAAEDAEELSHECKELLLSLPKERGWR 37
                          *. ..*. .:             . **: * ** * :*:* *********

AtST2a          TRYLYLFQGFWCQAKEIQAIMSFQKHFQSLENDVVLATIPKSGTTWLKALTFTILNRHRF 120
VVTC49995       TPHIYLYQGFWCQPKEIQAITNFQHHFQARDTDLILATMPKAGTTWLKAMAFAILNRK-- 95
                * ::**:******.****** .**:***: :.*::***:**:*******::*:****:
```

```
AtST2a      DPVASSTNHPLFTSNPHDLVPFFEYKLYANGDVPDLSGLASPRTFATHLPFGSLKETIEK 180
VVTC49995   -PIPVSTTHPLHTSNPHDLVPFLEYKLYANNNFPDLSKLPQPRLFATHVPFASLPESITK 154
             *:. **.***.**********:*******.:.**** *..** ****:**.** *:* *

AtST2a      PGVKVVYLCRNPFDTFISSWHYTNNIKSESVSPVLLDQAFDLYCRGVIGFGPFWEHMLGY 240
VVTC49995   SNSRVVYMCRNPLDAFASSWHFLMKARPESLGPISIEEGFQMYCKGSMAFGPFWKHMLGY 214
            .. :***:****:*:* ****:  : :.**:.*: :::.*::**:* :.*****:*****

AtST2a      WRESLKRPEKVFFLRYEDLKDDIETNLKRLATFLELPFTEEEERKGVVKAIAELCSFENL 300
VVTC49995   WKESIERPHKVLFMKYEDTKEDIIFQLKRLAKFLGVPFSLEEERKGVIEEIARLCSFENL 274
            *:**::**.**:*::*** *:**  :*****.** :**: *******:: **.*******

AtST2a      KKLEVNKSNKSIKNFENRFLFRKGEVSDWVNYLSPSQVERLSALVDDKLGGSGLTFRLS 359
VVTC49995   KNLEVNQSGKSIGYFENKDLFRKGVVGDWVNLLTPLMAKQLVQVMEEKLEGSDLSFKVF 333
            *:****:*.***  ***: ***** *.**** *:*  .::*  ::::** **.*:*::
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10. It is not necessary to disclose in the specification, plant transformation experiments using the present invention on each and every kind of plant species of interest. More importantly, such extensive experimentation would be unnecessary in view of the knowledge and methodologies which are within the routine skill of the artisan as evidenced herein.

11. Thus, on the basis of my personal knowledge, education and experience in this particular field of molecular biology, I am of the informed opinion that the present invention as claimed is fully enabled and described by the present specification.

12. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

November 15, 2006
Date

[signature]
Luc Varin

## ANNEX A

To illustrate the difference between the effect mediated by jasmonic acid and 12-hydroxyjasmonate on gene expression, selected results from an mRNA profiling experiment performed with the *A. thaliana* Affymetrix DNA chips are presented in Table 1, 2 and 3. It is important to note that only selected genes are presented. The Affymetrix DNA chips contain more that 22,000 entries and a large number of genes are clustering with the ones presented in the three Tables.

The results show clearly that jasmonic acid and 12-hydroxyjasmonate have different effects on gene expression. For example, 12-hydroxyjasmonate does not repress the expression of genes involved in photosynthesis (Table 1). Furthermore, 12-hydroxyjasmonate does not induce the expression of THI2.1 a marker gene in the plant defense response (Table 2) A similar result with *THI 2.1* was presented in the publication by Gidda. S *et al.* (2003) *J. Biol. Chem.* 278, 17895-17900.

The microarray results clearly show that 12-hydroxyjasmonate induces gene expression in *A. thaliana* and that this induction is independent of the jasmonic acid induction pathway (Table 3).

To summarize, the advantages of modulating the endogenous levels of 12-hydroxyjasmonate by sulfonation over knocking down the synthesis of jasmonic acid to control flowering time are:

- The absence of negative side effects on growth.
- The absence of negative side effects on the defense response.

**Table 1. Selected genes encoding enzymes involved in photosynthesis.**

| Accession | Control | methyl jasmonate* | 12-hydroxyjasmonate** |
|---|---|---|---|
| At1g44446 | 4164*** | 555 | 3810 |
| At5g01530 | 42663 | 29136 | 45635 |
| At4g27440 | 19087 | 4315 | 21581 |
| At1g29910 | 48449 | 27488 | 53212 |
| At1g19150 | 9037 | 6366 | 9204 |

* Plants were treated with 50 micromolar of methyl jasmonate for a period of four hours.
** Plants were treated with 50 micromolar of 12-hydroxyjasmonate for a period of four hours.
*** Numbers represent normalized gene expression values. Three independent biological replicates were used for this study.

**Table 2. Selected genes encoding proteins involved in the plant defense response**

| Accession | Control | methyl jasmonate | 12-hydroxyjasmonate |
|---|---|---|---|
| At5g07010 | 120 | 9744 | 223 |
| At3g55970 | 130 | 6956 | 90 |
| At1g63040 | 8 | 296 | 8 |
| At1g72260 | 104 | 3487 | 64 |
| At1g54020 | 71 | 2278 | 59 |
| At5g42900 | 73 | 1905 | 69 |
| At3g16330 | 16 | 363 | 16 |
| At3g20810 | 198 | 3950 | 168 |
| At5g37260 | 56 | 1105 | 50 |
| At2g22880 | 8 | 148 | 9 |
| At3g23550 | 247 | 4029 | 250 |

* Plants were treated with 50 micromolar of methyl jasmonate for a period of four hours.
** Plants were treated with 50 micromolar of 12-hydroxyjasmonate for a period of four hours.
*** Numbers represent normalized gene expression values. Three independent biological replicates were used for this study.

**Table 3. Selected genes induced by 12-hydroxyjasmonate**

| Accession | Control | methyl jasmonate | 12-hydroxyjasmonate |
|---|---|---|---|
| At1g30140 | 3 | 3 | 44 |
| At4g04070 | 4 | 7 | 73 |
| At5g21110 | 4 | 3 | 72 |

* Plants were treated with 50 micromolar of methyl jasmonate for a period of four hours.
** Plants were treated with 50 micromolar of 12-hydroxyjasmonate for a period of four hours.
*** Numbers represent normalized gene expression values. Three independent biological replicates were used for this study.